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Award Number: W81XWH-07-1-0437

TITLE: A Role for Ubiquitin Binding in Bcr-Abl Transformation

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REPORT DATE: June 2008

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
01-06-2008	Annual	1 JUN 2007 - 31 MAY 2008

4. TITLE AND SUBTITLE

A Role for Ubiquitin Binding in Bcr-Abl Transformation

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-07-1-0437

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

University of Medicine and Dentistry of New Jersey
Newark, NJ 07107-3001

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT
We have previously identified a docking site for ubiquitin in the amino-terminus of p210 BCR/ABL. Since ubiquitin binding has been implicated in both the intra- and inter-molecular regulation of many signaling molecules, this association may have important clinical consequences for chronic myelogenous leukemia. During Year 1 of this study we have precisely mapped the binding site for ubiquitin, and generated a p210 BCR/ABL mutant that is completely impaired in binding in mammalian cells. Loss of ubiquitin binding does not destabilize p210 BCR/ABL, thus allowing us to directly evaluate the mutant in assays for leukemogenic activity. This mutant will provide us with a unique opportunity to directly assess the importance of the ubiqutin-p210 BCR/ABL interaction in CML. These studies will be performed in Year 2 of this study.

15. SUBJECT TERMS
P210 BCR/ABL, Ubiquitin, Ubiquitin Binding Domain, Yeast 2-Hybrid Mapping, Animal Model For Chronic Myelogenous Leukemia

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	6	19b. TELEPHONE NUMBER *(include area code)*

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PROGRESS REPORT

Introduction:

BCR is a ubiquitously expressed protein that is found in both the nuclear and endosomal compartments. Although the native function of BCR is unknown, we have recently described two studies which indicate that the nuclear and cytoplasmic functions may be distinct (1,2). In the nucleus, BCR interacts with, and modulates the activity of, transcriptional regulators such as c-Myc and the Xeroderma pigmentosum type B (XPB) protein. In the cytoplasm, BCR interacts with subunits of the mammalian ESCRT I complex and regulates endosome-mediated growth factor receptor turnover. While studying the role of BCR in endosomal trafficking we made the surprising observation that BCR contains a ubiquitin binding domain within its NH_2-terminus, and that the structural integrity of this site is retained in p210 BCR/ABL. Although direct binding to ubiquitin is relatively uncommon, this association has been implicated in several distinct biological processes. Importantly, recent evidence suggests that this interaction can direct a proteins own monoubiquitylation, which in turn is thought to facilitate allosteric regulation. The importance of the ubiquitin docking site for BCR/ABL transformation is unclear, and is the central issue of this proposal. The goals of the proposal are relatively straightforward; to generate a mutant of p210 BCR/ABL that no longer interacts with ubiquitin, and determine whether the mutant is impaired in transforming activity. In Year 1 the goal was to generate and validate a binding mutant while in Year 2 the mutant would be examined in cell- and animal-based models for chronic myelogenous leukemia.

Body:

Task 1a: Map the docking site for ubiquitin by yeast 2-hybrid analysis (Months 1-6). We had previously determined by yeast 2-hybrid analysis that the ubiquitin docking site lies within the first 413 amino acids of BCR, and had hypothesized that it may be associated with a UBD consensus sequence that we had identified at residues 143-162. In the first 6 months of the study we used a yeast 2-hybrid approach to map the ubiquitin docking site. A series of truncation mutants was constructed in the pGBT9 yeast 2-hybrid vector, and each was tested individually for binding with full-length ubiquitin (Fig. 1). As a positive control for binding each construct was also tested for binding with the isolated oligomerization domain of BCR. Surprisingly, our analysis revealed that the ubiquitin docking site is contained within an interval (residues 178-192) that does not contain the putative UBD. An examination of this interval did not indicate any similarity with known UBDs suggesting that the UBD of BCR is unique. Since it has been previously reported that autophosphorylation of Tyr177 of p210 BCR/ABL creates a binding site for GRB2, we wondered whether ubiquitin may also bind to this residue. Thus, we constructed a Tyr177Ala mutant in full-length BCR and tested for binding with ubiquitin (Fig. 1). This mutant still bound to ubiquitin suggesting that the binding sites for GRB2 and ubiquitin are discrete and separable. This task is now complete.

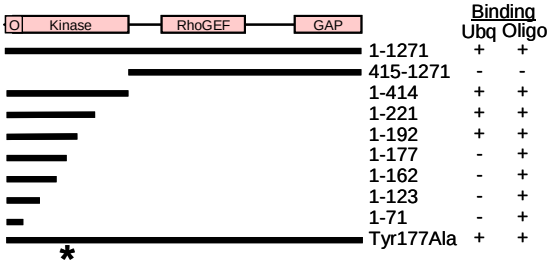

Figure 1: Mapping of the ubiquitin docking site within the NH_2-terminus of BCR. Yeast 2-hybrid analysis was used to map the ubiquitin binding site to residues 178-192 of BCR. Numbers indicate amino acid residues contained within BCR derivatives that were tested for binding with full-length ubiquitin (Ubq). All constructs were also tested against the isolated oligomerization domain of Bcr (Oligo) which served as a positive control for binding. Asterisk indicates the position of a tyrosine to alanine substitution in residue 177.

Task 1b: Construct and validate a ubiquitin binding mutant for p210 BCR/ABL (months 6-12). Once the docking site for ubiquitin was identified, site directed mutagenesis was used to delete the site in the context of full-length p210 BCR/ABL. The mutant was constructed in the backbone of the yeast 2-hybrid vector pGBT9 and then tested for binding with ubiquitin and XPB (Fig. 2). XPB is a known binding partner for BCR and

p210 BCR/ABL that interacts with the centrally located RhoGEF domain (see upper schematic in Fig. 1). It was included as a positive control to validate the structural integrity of the mutant. As expected the ubiquitin binding mutant fails to interact with ubiquitin, but still interacts with XPB. In contrast, p210 BCR/ABL interacts with both ubiquitin and XPB. This observation suggests that we have generated a structurally intact binding mutant for ubiquitin, and that p210 BCR/ABL contains a single docking site for ubiquitin. This task is now complete.

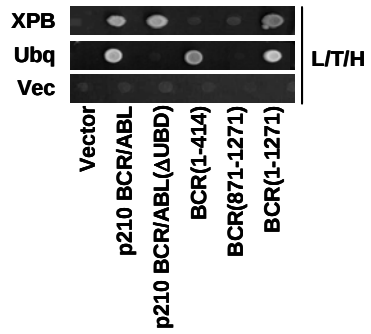

Figure 2: Characterization of a ubiquitin binding mutant of p210 BCR/ABL in yeast. Yeast colonies that grew on plates lacking leucine and tryptophan were examined for growth on histidine deficient plates (L/T/H). Interactions between proteins are demonstrated by the ability to activate the *HIS3* reporter gene

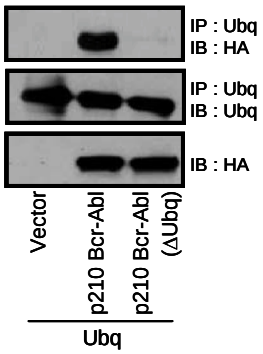

Figure 3: Construction of a p210 BCR/ABL mutant that is impaired in ubiquitin binding in mammalian cells. The HA-tagged p210 BCR/ABL(ΔUbq) mutant does not interact with ubiquitin. 293T cells were co-transfected with the indicated combinations of plasmids. IP indicates antibody used for immunoprecipitation and IB indicates antibody used for immunoblot.

Task 1c: Characterize the binding mutant in mammalian cells (months 12-15). The next set of experiments in this study required that we validate and characterize a ubiquitin binding mutant in mammalian cells. For this analysis, the ubiquitin docking site was deleted by site-directed mutagenesis in the mammalian expression construct pAX142-p210BCR/ABL (pAX142-p210BCR/ABL(ΔUbq)). We then transiently co-expressed ubiquitin along with p210 BCR/ABL, p210BCR/ABL(ΔUbq), or cognate vector in 293T cells and performed a co-immunoprecipitation to detect an interaction (Fig. 3). As shown, we were readily able to co-immunoprecipitate ubiquitin with p210 BCR/ABL, but not with the ubiquitin binding mutant. Over the next several months we anticipate that we will be able to thoroughly characterize this mutant for kinase and binding activity. We are ahead of schedule on this task.

Tasks 2: Determine whether ubiquitin binding is required for p210 BCR/ABL transformation (months 9-24). Task 2 requires that we introduce the ubiquitin binding mutant into the MIG, bicistronic retroviral vector, and then examine the construct in cell- and animal-based models for p210 BCR/ABL transformation. Because of the size of the vector and insert, this mutant cannot be made by site-directed mutagenesis. It involves a challenging cloning strategy that requires several intermediate steps. However, we have previously used this strategy to generate mutants of p210 BCR/ABL that lack XPB-binding and RhoGEF activity, and do not anticipate any technical difficulties. This cloning project is underway and we expect to have the construct ready for analysis within the next 4-6 weeks. This task is ongoing.

Key Research Accomplishments

- We have identified the docking site for ubiquitin within the amino-terminus of BCR and p210 BCR/ABL.

- We have determined that p210 BCR/ABL contains a single docking site for ubiquitin.
- We have generated and validated a ubiquitin binding mutant of p210 BCR/ABL in a mammalian expression construct.

Reportable Outcomes

None

Conclusion

During Year 1 of this study we have identified the docking site for ubiquitin within the amino-terminus of p210 BCR/ABL and generated a mutant that lacks this site. We have also determined that p210 BCR/ABL contains a single docking site, which is unique for ubiquitin binding proteins. Importantly, loss of ubiquitin binding does not affect the stability of p210 BCR/ABL which will allow us to proceed with our cell- and animal-based studies of transformation. Within the next 6-12 months we will now be able to clearly determine whether ubiquitin binding is required to support p210 BCR/ABL transformation. If this is the case, this would suggest that this interaction may be a viable target for therapeutic intervention in the treatment of chronic myelogenous leukemia.

References

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